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(54) CORNEE ET SCLEROTIQUE ARTIFICIELLES IN VITRO
(54) IN VITRO ARTIFICIAL CORNEA AND SCLERA

(57)

The invention provides an in vitro avascular human corneal equivalent that comprises immortalized human cell lines. As these corneal equivalents are in vitro models, they are maintained in an incubator throughout the testing period, thereby eliminating the problems and expense associated with animal care. The corneal equivalent is preferably surrounded by a matrix in which angiogenesis (formation of capillary-like structures) can occur in vitro. This surrounding matrix has the potential to play the role of a pseudo-sclera, allowing the in vitro assessment of the cornea's angiogenic reaction to any substance or injury. Furthermore, the model is capable of being produced easily, is physiologically functional and can give predictable and quantifiable results when submitted to various drugs, chemicals and/or physical trauma. Modifications can be made, such as the use of primary donor cells instead of cell lines; and the physical and chemical treatments of the matrix material to make the constructs suitable for use in transplantation.

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(54) **CORNEE ET SCLEROTIQUE ARTIFICIELLES IN VITRO**
(54) **IN VITRO ARTIFICIAL CORNEA AND SCLERA**

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ABSTRACT

The invention provides an *in vitro* avascular human corneal equivalent that comprises immortalized human cell lines. As these corneal equivalents are *in vitro* models, they are maintained in an incubator throughout the testing period, thereby eliminating the problems and expense associated with animal care. The corneal equivalent is preferably surrounded by a matrix in which angiogenesis (formation of capillary-like structures) can occur *in vitro*. This surrounding matrix has the potential to play the role of a pseudo-sclera, allowing the *in vitro* assessment of the cornea's angiogenic reaction to any substance or injury. Furthermore, the model is capable of being produced easily, is physiologically functional and can give predictable and quantifiable results when submitted to various drugs, chemicals and/or physical trauma. Modifications can be made, such as the use of primary donor cells instead of cell lines; and the physical and chemical treatments of the matrix material to make the constructs suitable for use in transplantation.

IN VITRO ARTIFICIAL CORNEA AND SCLERA

FIELD OF THE INVENTION

The invention is in the field of tissue engineering and culture systems and is directed primarily towards reconstruction of *in vitro* cell-based models for use as animal alternatives in irritancy, toxicity and drug efficacy testing. In particular, the invention relates to an artificial cornea.

BACKGROUND OF THE INVENTION

Animal tests are used for screening new chemicals, drugs, finished products or their ingredients for potential ocular irritancy. The historic test that is still in widespread use is the Draize rabbit eye irritancy test. The Draize test was developed in 1944 and involves the introduction of 0.1 ml of a test substance into the lower conjunctival sac of albino rabbit eyes (Draize *et al.* 1944). Responses of the cornea, conjunctiva and iris are graded using a numerical scoring system. The cornea is scored for opacity that is induced by irritants. The conjuctivum is scored for an angiogenic response (such as redness caused by blood vessel dilation) while the iris gives a neurological response (i.e., contraction or dilation). Of these, an indication of corneal clarity/opacity is the most relied upon indicator for irritancy. Although still regarded as the definitive test available, the Draize test suffers not only from strong resistance from the public, but also because the rabbit eye responds differently to various traumas than does the human eye. Animal testing is also used in the development of ophthalmic drugs. Again, differential responses between animal models such as the rabbit and humans are well documented for drugs ranging from analgesics for pain, antibiotics, to steroids and non-steroid preparations that modify wound-healing responses.

Enucleated whole eyeballs from rabbits and cattle have been tested as possible Draize alternatives. Among them are the Bovine Eye Assay which measures corneal

opacity/permeability from Merck Sharp and Dome laboratories. While enucleated rabbit and bovine eyes have shown a capacity to handle and identify severely irritating substances over a wide range of physical forms and solubilities, they are less sensitive for resolving responses to very mild and moderately irritating substances (Burton *et al.* 1981; Gautheron *et al.* 1992). In addition, these are animal derived tissues and therefore have the same accuracy problems and difficulty with extrapolation to humans as the Draize test. Moreover, the very short term viability of these corneas (tests are done on the day of enucleation) will not allow for longer term screening of more chronic effects of the milder test substances.

Organ cultured human corneas can be used for testing. Richard *et al.* (1991), Anderson *et al.* (1993) and Collin *et al.* (1995) have successfully organ cultured freshly obtained whole human corneas, allowing for investigations to be carried out on whole corneas. However, organ cultured corneas have a finite lifespan. The longest time recorded is 21 days (Anderson *et al.* 1993). More importantly, the availability of donors is sporadic and donor corneas are too heterogeneous to be useful as a practical model for drug testing.

Other alternatives that have been developed include the EYTEX™ test from In Vitro International (formerly Ropak Laboratories) which is based on the quantification of opacity produced in a synthetic protein matrix on exposure to chemical irritants (Gordon, 1992); the Keratinocyte Neutral Red Uptake Bioassay from Clonetics Corporation based on the methodology of Borenfreund and Puerner (1985), the MTT assay using living skin equivalent from Advanced Tissue Sciences (Osborne *et al.* 1995), and the Chorioallantoic membrane vascular assay (Bagley et al, 1992). Trans-epithelial permeability assay can also be used as *in vitro* assay for predicting ocular irritancy using MDCK cells (Martin and Stott, 1992). Others use an agar diffusion cytolysis as alternative screen for the prediction of a corrosive ocular response using rabbit cornea fibroblasts (Galer, 1992). Results indicate that while it may not be possible to use a single test to classify compounds as to irritation potential, some of the alternative test models may be useful in prioritizing and reducing the number of in vivo irritation tests conducted. However, all of the tests are dependent upon continued rabbit eye testing as definitive tests and also to develop validation databases to aid interpretation of the *in vitro* data.

Researchers have been attempting to reconstruct corneas *in vitro* from cell lines. Individual human corneal epithelial layers have been successfully maintained in culture as a stratified epithelium by Kahn *et al.* (1993) and Araki-Sasaki *et al.* (1995). These investigators had used corneal epithelial cell lines that were immortalized using a hybrid SV40-adenovirus. Successful reconstructions of corneas comprising the three main layers have also been recently reported by Minami *et al.* (1993) and Zieske *et al.* (1994). These corneas were reconstructed from either passaged primary cells from bovine (Minami *et al.* 1993), or mixed cultures of primary rabbit epithelial and stromal cells and an immortalized mouse endothelial cell line (Zieske *et al.* 1994).

To date, there have been no reports of successfully reconstructed human corneas based fully on human cell lines while mimicking the physiology of the human cornea and surrounding tissue.

SUMMARY OF THE INVENTION

The present invention provides an artificial mammalian cornea which comprises:

a) an endothelium comprising primary or immortalized mammalian endothelium cells;

b) a stromal matrix;

c) an epithelium comprising primary or immortalized mammalian epithelium cells; and

d) at least one layer selected from Bowman's membrane and Descemet's membrane.

The present invention also provides a method for preparing an artificial mammalian cornea which comprises:

a) growing an endothelium which comprises primary or immortalized mammalian endothelium cells;

b) optionally layering a Descemet's membrane on the endothelium;

c) forming a stromal matrix on the endothelium or on the Descemet's membrane, if present;

d) optionally layering a Bowman's membrane on the stromal matrix; and

e) growing an epithelium, which comprises primary or immortalized mammalian epithelium cells, on the stromal matrix or on the Bowman's membrane, if present;

wherein at least one of Bowman's membrane and Descemet's membrane is present.

The present invention further provides a method for preparing an artificial mammalian cornea which comprises:

a) growing an epithelium which comprises primary or immortalized mammalian epithelium cells;

b) optionally layering a Bowman's membrane on the epithelium;

c) forming a stromal matrix on the epithelium or on the Bowman's membrane, if present;

d) optionally layering a Descemet's membrane on the stromal matrix; and

e) growing an endothelium, which comprises primary or immortalized mammalian endothelium cells, on the stromal matrix or on the Descemet's membrane, if present;

wherein at least one of Bowman's membrane and Decemet's membrane is present.

The present invention aslo provides a commercial package which comprises:

a) the cornea as described herein, and

b) instructions for its use for testing a substance for ocular irritancy, toxicity or drug efficacy, or transplantation.

The present invention also provides a serum-free culture medium which comprises: at least one of a protease inhibitor, a growth factor, a substance which mediates the growth factor, a substance which prevents cellular contraction and a free-radical scavenger.

The present invention also provides an artificial sclera which comprises primary or immortalized mammalian angiogenic cells.

Gene transfer and tissue culture techniques have been used to construct a cornea from immortalized human cell lines that is analogous to the human cornea *in vivo*. The corneal equivalent is preferentially surrounded by a sclera that will be able to mimic inflammation responses. The matrix comprising a combination of extracellular matrix molecules and growth factors allows reconstruction of other body tissues that could be used as complementary *in vitro* models for alternative models to animal testing.

Various different gene constructs have been introduced into the cell lines by transfection and/or retroviral infection to either overexpress, or block expression (antisense constructs) of, gene products in order to mimic different pathological conditions that occur in the cornea. This allows the development of *in vitro* corneal disease models for studying drug treatment efficacy.

The model can also be modified to render it suitable for use in transplantation. We have also developed a medium that can be used to start and maintain cultures of primary cells and cell lines from cornea and other tissues. The medium is also suitable for the culture of the reconstructed model in the absence of serum.

The artificial cornea is preferably surrounded by a matrix in which angiogenesis (formation of capillary-like structures) can occur *in vitro*. The cornea is physiologically and functionally similar to the human cornea and has the potential to respond to ocular irritants, drugs and injuries. The surrounding angiogenic material plays the role of pseudo-sclera and thereby provides the ability to assess *in vitro* the angiogenic reaction to any chemical irritants, drugs or any injuries. The invention extends to a method for making the artificial cornea and a method for using the artificial cornea. Preferably, the corneal or scleral cells used, either primary or immortalized, are of human origin.

The invention also provides a serum-free medium that we have used to successfully start and maintain the cell lines and reconstructed model. The matrix comprising a combination of extracellular matrix molecules and growth factors allows reconstruction of other body tissues that could be used as complementary *in vitro* models for alternative models to animal testing.

The reconstructed cornea can also be modified for transplantation by modification of the matrix compounds by physical and chemical treatments, and incorporation of matrix proteins or essential amino acid sequences and/or growth factors. Primary cells can be used until the safety of cells containing viral constructs for transplantation has been determined. In addition, cells from one or more layers may be omitted to allow for the host/patient's cells to repopulate the transplanted matrix material.

DETAILED DESCRIPTION OF THE INVENTION

The invention comprises mammalian, preferably human, corneal cell lines, either primary or immortalized, that have been reconstructed to mimic the human cornea in structure, physiological functioning and responses to drugs, chemicals and other compounds. The reconstructed cornea can be constructed on its own or with a surrounding sclera. The latter is preferred when testing of angiogenic responses are required (e.g. in mimicking conditions when vascular invasion of the cornea occurs due to irritation or injury from physical, chemical sources; or disease). The reconstructed cornea with/without sclera can be cultivated either in serum-supplemented medium or a serum-free medium such as the one we developed and describe below.

Corneal cell lines

Cell lines were produced from each of the main corneal layers: epithelium, stroma and endothelium. The epithelial and endothelial layers were isolated by scraping post-mortem human corneas with a surgical Gill knife. Pieces from each of these layers were placed separately in culture medium containing a 1:1 mixture of Medium 199 (GIBCO, Burlington, Canada): Optisol (Chiron Ophthalmics, Irvine, CA) supplemented with 10% foetal bovine serum (FBS; GIBCO), 1% insulin-transferrin-selenium (ITS; Becton Dickinson Canada, Mississauga, Canada) and 0.1% gentamicin (GIBCO). The serum-free medium developed, and disclosed herein, can also be used, as well as any conventional serum-based or serum-free growth medium. The pieces were dissociated by pipetting up and down and the cells were left to grow out onto the tissue culture dish. The stroma, which was scraped clean of residual epithelium and endothelium was cut into pieces and digested with 0.25% collagenase at 37°C for several hours to loosen the matrix and release stromal keratocytes. The stromal pieces are further broken down by either pipetting or passing through a syringe. The collagenase is removed by aspiration after centrifugation of the mixture. The dissociated stroma is then plated onto tissue culture dishes and supplemented with the medium described above.

The cells take from two days to over a week to settle and multiply. After colonies are present , the medium is aspirated and replaced with Medium 199, 10% FBS and 1% ITS . Colonies of outgrowiing cells with the appropriate morphology for the cell type are picked out and expanded. These are then used for producing immortalized cell lines.

Cells were immortalized in two ways. Some primary cells (endothelial, stromal, epithelial) were transfected with a combination of plasmids that contained the SV40 large T antigen, pSV3neo (Southern and Berg, 1982) and the AD5-E1A region (gift from Dr. Steve Whelan), using standard calcium phosphate transfection, using a BES buffer. After transfection, the cells were returned to their regular medium (M199, 10% FBS, 1% ITS) and then passage just before confluence. Resistance to G418 was used to select for successful transfectants. For corneal cells, 1mg/ml G418 was added to the culture medium until the control cells died.

Other cells (epithelial, stromal) were infected with an amphotropic recombinant retrovirus that contains HPV16 genes E6 and E7 (Halbert et al., 1991, 1992). The producer cell line PA317LXSN 16E6E7 was obtained from the ATCC. Essentially the corneal cells were infected with serially diluted viral-containing conditioned culture supernatants from the producer cells in the presence of 10 μg/ml polybrene (Sigma, St. Louis, MO, USA). After 24 hours of exposure to the virus, the viral supernatant was removed and the cells cultured, passaged once and then immortalized cells were selected for by G418 resistance.

All three types of cell lines were initially screened using morphological criteria. The cell lines that showed the appropriate morphology were then screened using electrophysiology for functional similarities to the cells of the human cornea, preferably according to the methods of Rae et al. (1991) or Watsky et al. (1992). Cells that were similar to corneal cells morphologically and physiologically were screened for expression of the appropriate biochemical markers. Cells that were deemed similar to those of the cornea were then given a final test by performing an initial reconstruction of the cornea only.

Morphological and biochemical markers

Epithelial and endothelial cells were initially selected for by their cobblestone morphology at confluence. Stromal keratocytes are characteristically fibroblastic and form

whorls at confluence. After this initial screening, cells were subjected to immunohistochemical staining for expressed adenovirus E1A or large T antigen. Cells that expressed these proteins were then screened electrophysiologically for similarities to cells that were freshly dissociated from post-mortem corneas and from low passage primary cultures, as described below. Following the electrophysiological screening, the cells were then screened for expression of the following markers. Epithelial cells were stained with the AE5 antibody (ICN) that recognises corneal specific keratin 3, a 64kD protein found in differentiated corneal epithelial cells (Schermer et al. 1986). Keratocytes were stained for vimentin, an intermediate filament which is used as a marker for fibroblastic cells, using an antibody from Sigma. Endothelial cells were probed by *in situ* hybridisation for expression of alpha2(VIII) collagen, which is produced by the corneal endothelium and is a component of Descemet's membrane.

Reconstruction of the cornea

The method used is a modification of the protocol described in Zieske et al. (1994). In the present reconstruction method, the insert used is preferably an open-topped plastic cylinder with a semi-porous membrane base. More preferably, either a Costar transwell insert that is pre-coated with type I and type III collagen, or a Milllicell-CM (Millipore) culture insert is used. The latter inserts were coated, preferentially by airbrushing, with type I collagen (Collaborative) in 60% ethanol. For the latter, the coated inserts are air dried and washed with medium several times to neutralize the acidity of the collagen preparation.

Corneal endothelial cells in culture were trypsinized, re-suspended in complete Medium 199 (M199 + 10% FBS + 1% ITS + gentamicin) and plated onto the collagen coated insert at a density of 3.0×10^5 cells per ml. The cells were allowed to grow until about 80% confluence.

Descemet's membrane, which in this case was a mixture of type I and IV collagen and fibronectin in collagen medium (comprising M199, HEPES buffer (200 mM HEPES, 100 mM NaOH), FBS and gentamicin) was then layered on top of the layer of endothelial cells. After this mixture had gelled, the stroma, which is a mixture of type I collagen, 1.5% chondroitin sulphate (other proteoglycans can be substituted to give different consistencies and transparency) in the

collagen medium containing 7.5 x 10^4 keratocytes per ml was layered on top. 1.3 ml of stroma was placed into each insert. The stroma was then allowed to set in the 37°C incubator for 20 min.

Bowman's membrane was then added. This consisted of 150 µl of neutralized type I collagen with 0.1 mg/ml fibronectin and 0.1 mg/ml laminin per insert that was added on top of the stroma and allowed to set.

Finally, 3.0 x 10^5 cells per ml epithelial cells were plated on top of the collagen matrix. The culture inserts containing the reconstituted corneas were then cultured until the epithelial cells reached confluence. This usually took one week. Once the reconstruction was completed, the corneas were cultured in serum-free medium. The serum-free medium comprises at least one of a protease inhibitor, such as aprotinin; a cell growth promotor that also prevents cellular contraction, such as retinol acetate; a growth factor, such as EGF or bFGF; a substance which mediates the growth factor, such as a proteoglycan, preferably chondroitin sulphate; a protein such as heparin; and a free radical scavenger, such as superoxide dismutase. A preferred serum-free medium, as shown in Table 1, was used.

TABLE 1. Composition of 2X stock for serum-free medium.

DMEM	94 ml
ITS	2 ml (1%)
Dextran	2 g (1%)
Albumax	0.6 g (3 mg/ml)
Chondroitin Sulphate C	2.7 g (1.35%)
Sodium heparin	0.018 g
Glutamax-1	2 ml
Aprotinin	0.14 TIU
Hydrocortisone (4 mM)	10 µl
Putrescine (100mM)	200 µl
Progesterone (200 mM)	20 µl
Retinol Acetate (5 mM)	200 µl
EGF (10 mg/ml)	200 µl
bFGF (50 µg/ml)	200 µl
SOD (5 mg/ml)	100µl
Gentamicin or antibiotic-antimycotic	200 ml

I. Make up 1X DMEM medium.

II. Add dextran, Albumax, ITS, sodium heparin, Glutamax and chondroitin C. If necessary, warm gently to dissolve reagents. Filter sterilize.

III. Just before use, add remaining constituents using filter sterilized 1000X stock solutions. (Keep retinol acetate protected from light.)

IV. Dilute with 1:1 with 1X DMEM (without anything added).

NB. Modifications of the medium by addition/removal of components allows adaptation for culture of other human and animal cells and cell lines.

The above serum-free medium is also able to sustain whole post-mortem corneas in organ culture for at least three weeks and maintain cells in monolayer cultures.

After the epithelial cells attained confluence, the medium on top of the cornea was removed to create an air-liquid interphase. Cell lines that were able to form a 5-layered cornea resembling the human cornea were expanded for use in the corneal-sclera reconstructions.

An alternative method used is to coat the insert with a collagen or fibrin matrix and then seed the epithelial cells, followed by the stroma after epithelial confluence and then the endothelium. Bowman's membrane and/or Descemet's membrane are applied after constructing the epithelial layer and stromal layer, respectively. Air-liquid interface is achieved by withdrawing medium from around the insert and allowing the epithelium to stratify, while maintaining the media supply to the construct from within the insert. This method is preferred when culture time is a factor, because epithelial confluence can be achieved in 1 to 3 days, depending on seeding density, instead of 1 week. Stromal and endothelial cell growth occur concurrently with epithelial stratification.

Vascular endothelial cell lines

Human umbilical vein endothelial cells (HUVECs) were harvested from umbilical cord veins as previously described (*Sirois E, et al., Int. J. Artificial Organs 16:609-619, 1993.).* They were grown on a gelatin-coated dish until confluence in Medium 199 supplemented with 20% FBS, 90 mg/l of heparin, 2 mM of L-glutamine and 100 µg/ml endothelial cell growth supplement (ECGS). Penicillin (100 I.U./ml), streptomycin sulfate (100µg/ml) and amphotericin B (0.25µg/ml) were present in medium. After the first passage, FBS concentration was reduced to 10% and ECGS to 20 µg/ml. Cells were subcultured at a ratio of 1:3 or 1:4 and HUVECs from the 2nd to 4th passages were used in our bioassays. Endothelial cell phenotype was verified by the diI-acetylated low-density lipoprotein (diI-Ac-LDL) uptake and positivity to factor VIII-related antigen antibodies by immunocytochemistry. Cultures were incubated in 5% CO_2 atmosphere at 37°C in saturated humidity. The medium was changed every other day.

For transfection, primary HUVECs were seeded onto uncoated 60 mm tissue culture plates and cultured until 60-70% confluent. They were then immortalized by infection with the HPV16 E6 and E7 genes and selected by G418 resistance as described above.

The virally transfected HUVECs were tested for functionality by their ability to undergo angiogenesis in a three-dimensional fibrin-containing matrix; and their ability to sequester acetylated low density lipoprotein labelled with 1, 1'-dioctadecyl -3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL; Biomed. Technol. Inc., Stoughton, MA).

Reconstruction of the cornea and sclera

The cornea complete with a surrounding sclera can be reconstructed in a variety of ways, using a range of cellular concentrations. Described below are two methods that have been optimised:

Method 1:

(Summarized in Figure 1)

As for the cornea alone, preferably either Costar transwell inserts that are pre-coated with type I and type III collagen, or collagen-coated Milllicell-CM (Millipore) culture inserts were used. A circular piece of agar or fibrin gel was placed in the centre of the culture insert and HUVECs were seeded at a density of 5 x 10^4 cells per ml and allowed to grow to confluence, supplemented by culture medium that had 0.09 g/l heparin, 0.02 mg/ml endothelial cell growth supplement (ECGS, Becton Dickinson).

Stromal and endothelial samples were simultaneously prepared and timed to have contracted at the same time as the HUVECs reach confluence. Stromal samples comprise keratocytes embedded in a collagen-chondroitin sulphate matrix as described previously. Retinol acetate was added to the stromal matrix to prevent contraction of the gel. Descemet's membrane was laid down on top of the stromal matrix and endothelial cells were seeded onto this membrane at 10^3 cells/ml. When the HUVECs attained confluence, the agar or fibrin gel was removed and the stroma and endothelial compartments were placed in the centre of the dish with the endothelial cells at the bottom.

The sclera, which was a combination of 10^5 to 10^7 vascular endothelial cells in a 0.3% to 1% fibrinogen (optimal is 0.3%) (fraction I; type I-S from bovine plasma (human fibrinogen can also be substituted)), 0.2% -1% (optimal is 0.25%) gelatin matrix, polymerized by addition of thrombin (various doses can be used, higher doses give a firmer gel) was then poured around the stroma and allowed to set. Bowman's membrane was then layered on top as described above and finally, epithelial cells were seeded on top of Bowman's membrane.

Method 2:

(Summarized in Figure 2)

Epithelial cells are plated on a collagen coated insert and grown until confluence is achieved (Fig. 2a). A Bowman's membrane is then applied on the epithelial layer. The insert membrane can also be used as a "pseudo-Bowman's membrane" by infiltrating with collagen and seeding the epithelial cells onto the underside of an inverted insert (Fig. 2b) and growing the cells until confluence, after which the insert is returned to its regular position. The stroma which had been fabricated separately as described in "Method One" is placed on top of the epithelial layer and surrounded with HUVEC cells (10^5 to 10^7 cells/ml matrix) in the fibrinogen matrix. Descemet's membrane is layered on top of the stroma and HUVEC/fibrinogen matrix. Endothelial cells are then seeded on top. The inside of the insert is then supplemented with medium while the epithelial layer is left to grow at a liquid-air interphase. This method reduces the culture time by up to one week (or the time required for the epithelial cells to achieve confluence on top of "Bowman's membrane").

During the assays in matrices, cells were grown in serum (10%) supplemented-medium 199 containing heparin, L-glutamine and antibiotics at the same concentration as described above, without ECGS, but with 50 ng/ml of FGF-2. Serum-free medium has also been used.

ALTERNATIVE METHODS FOR RECONSTRUCTION

1. The matrix base of the "stroma" can also be constructed from gelatin (a denatured collagenous material) instead of collagen I, chondroitin sulphate and other matrix components described above.

2. The fibrin, gelatin matrix of the "sclera" can also be modified for growing the keratocytes of the stroma. This matrix can replace the collagen-based matrix described above.

3. Other non-biological polymers can also be used instead of, or together with, the natural biopolymers. For example, 0.5% to 1% polyvinylpyrollidone (PVP) can be added to the fibrin/thrombin matrix described above in order to strengthen it.

Numerous modifications, variations and adaptations may be made to the particular embodiments of the invention described above without departing from the scope of the invention as defined in the claims.

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We claim:

1. An artificial mammalian cornea which comprises:
   a) an endothelium comprising primary or immortalized mammalian endothelium cells;
   b) a stromal matrix;
   c) an epithelium comprising primary or immortalized mammalian epithelium cells; and
   d) at least one layer selected from Bowman's membrane and Descemet's membrane.

2. The cornea of claim 1 wherein the stromal matrix comprises primary or immortalized mammalian stroma cells.

3. An artificial mammalian cornea which comprises:
   a) a stromal matrix;
   b) Bowman's membrane; and
   c) Descemet's membrane.

4. The cornea of claim 1 or claim 2, wherein Bowman's membrane and Descemet's membrane are present.

5. The cornea of any one of claims 1 to 4, which further comprises an artificial sclera comprising primary or immortalized mammalian angiogenic cells.

6. The cornea of claim 5, wherein the human angiogenic cells are human umbilical vein endothelial cells (HUVECs).

7. The cornea of any one of claims 1 to 6 wherein the cornea is an artificial human cornea.

8. A method for preparing an artificial mammalian cornea which comprises:

a) growing an endothelium which comprises primary or immortalized mammalian endothelium cells;

b) optionally layering a Descemet's membrane on the endothelium;

c) forming a stromal matrix on the endothelium or on the Descemet's membrane, if present;

d) optionally layering a Bowman's membrane on the stromal matrix; and

e) growing an epithelium, which comprises primary or immortalized mammalian epithelium cells, on the stromal matrix or on the Bowman's membrane, if present;

wherein at least one of Bowman's membrane and Descemet's membrane is present.

9. A method for preparing an artificial mammalian cornea which comprises:

a) growing an epithelium which comprises primary or immortalized mammalian epithelium cells;

b) optionally layering a Bowman's membrane on the epithelium;

c) forming a stromal matrix on the epithelium or on the Bowman's membrane, if present;

d) optionally layering a Descemet's membrane on the stromal matrix; and

e) growing an endothelium, which comprises primary or immortalized mammalian endothelium cells, on the stromal matrix or on the Descemet's membrane, if present;

wherein at least one of Bowman's membrane and Decemet's membrane is present..

10. The method of claim 8, which includes:

i) growing an artificial sclera, which comprises primary or immortalized mammalian angiogenic cells, around a resilient removable plug;

ii) removing the plug and inserting the endothelium, the optional Descemet's membrane and the stromal matrix, wherein the stromal matrix is essentially co-planar with the artificial sclera to receive the optional Bowman's membrane;

iii) optionally layering the Bowman's membrane on the stromal matrix and the artificial sclera;

iv) growing the epithelium on the stromal matrix and sclera or the Bowman's membrane, if present.

11. The method of claim 9, which includes, in step c, additionally forming an artificial sclera co-planar with the stromal matrix which artificial sclera comprises primary or immortalized mammalian angiogenic cells.

12. A commercial package which comprises:

a) the cornea of any one of claims 1 to 7, and

b) instructions for its use for either testing a substance for ocular irritancy, toxicity or drug efficacy, or transplantation..

13. A serum-free culture medium which comprises:
at least one of a protease inhibitor, a growth factor, a substance which mediates the growth factor, a substance which prevents cellular contraction and a free-radical scavenger.

14. An artificial sclera which comprises primary or immortalized mammalian angiogenic cells.

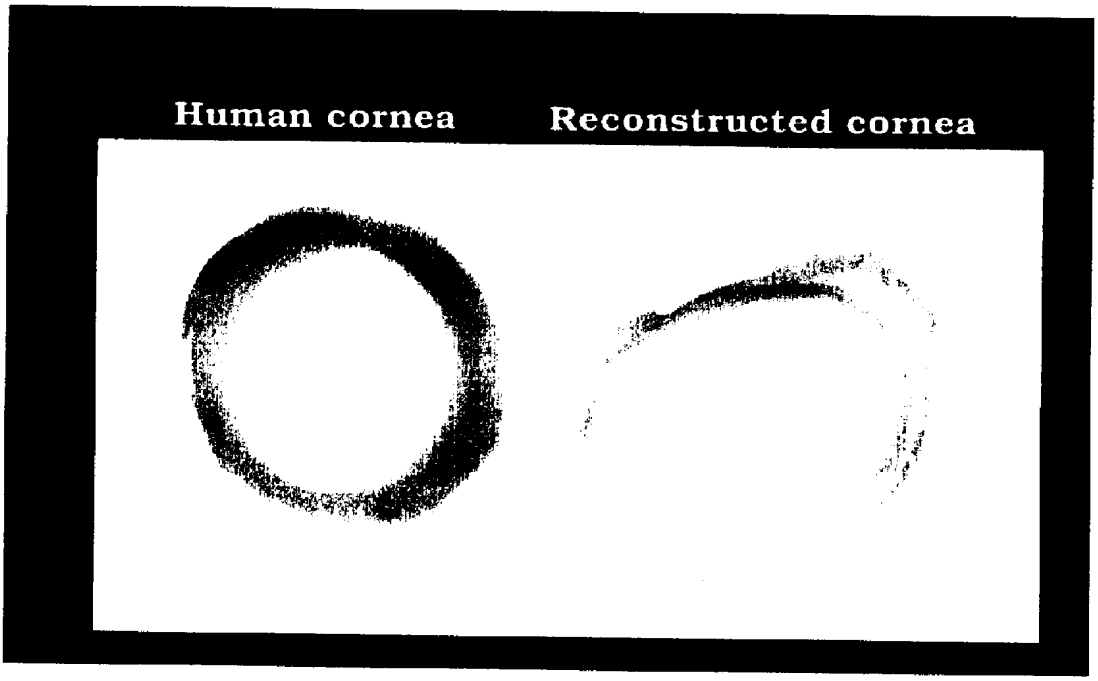

Fig. 3. Human cornea obtained from the Eye Bank and a cornea that was reconstructed from cell lines. Both were organ cultured in serum-free medium for one week.

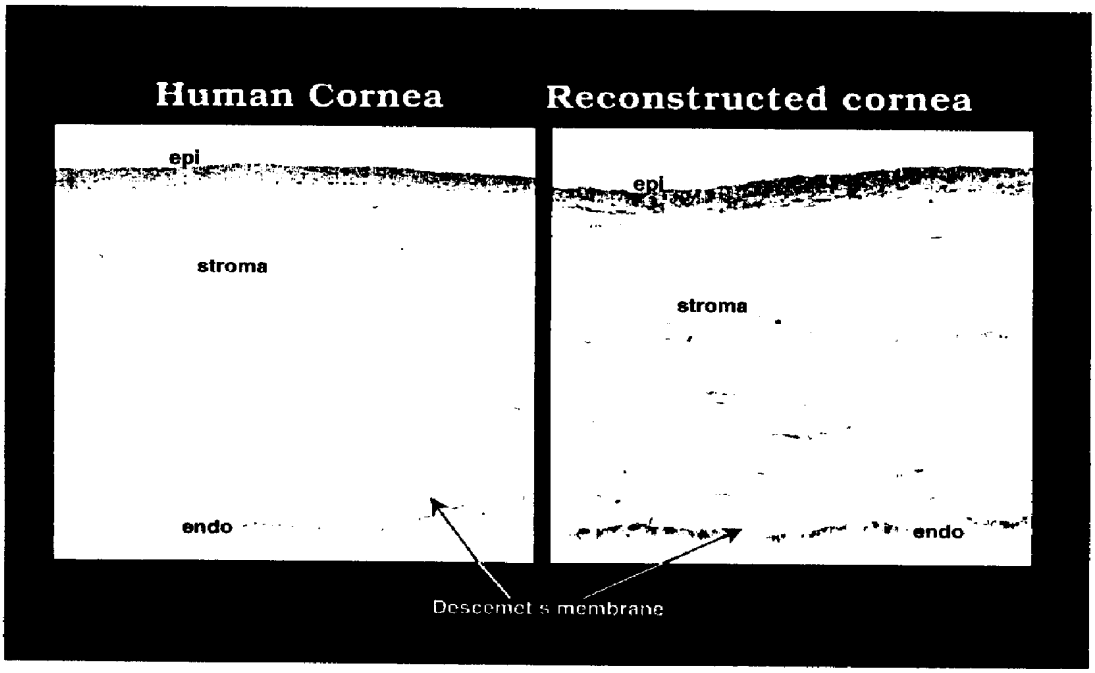

Fig. 4. Sections through a reconstructed cornea compared with an organ-cultured human cornea. Both show well defined epithelial, stromal and endothelial layers. Descemet's membrane is present in both specimens. However, Bowman's layer is not prominent in either specimen as seen in other samples of organ cultured human corneas.

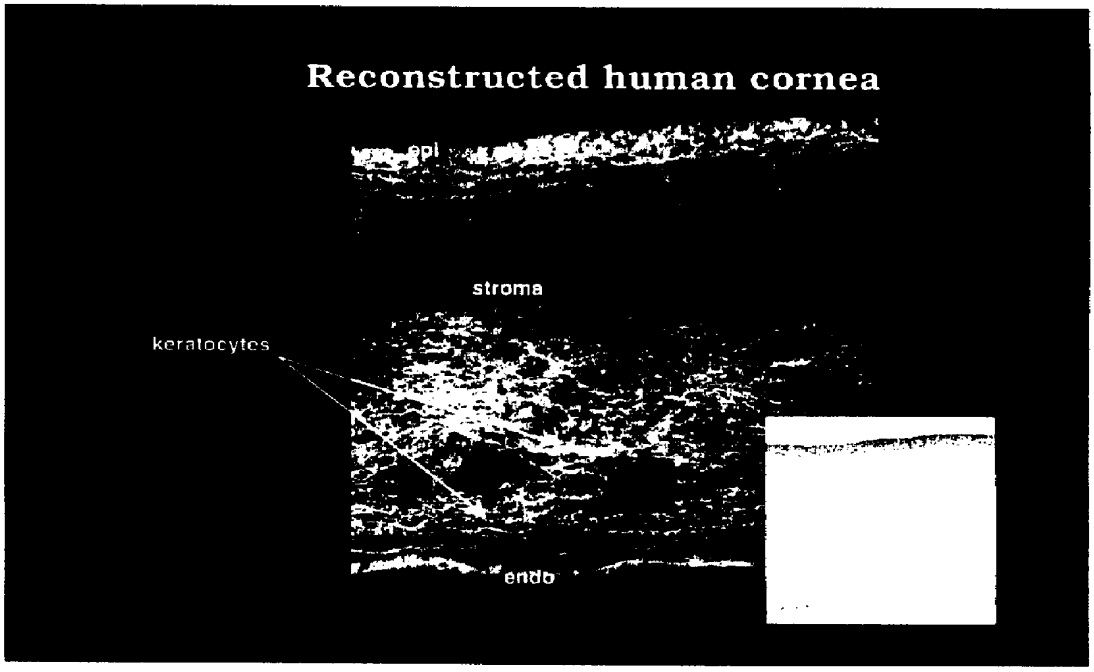

Fig. 5. Section through a reconstructed human cornea visualized with epifluorescence by confocal microscopy. The epithelial and endothelial layers are distinct, and keratocytes are present in the collagenous stromal matrix. Inset: H&E-stained section of the same cornea.

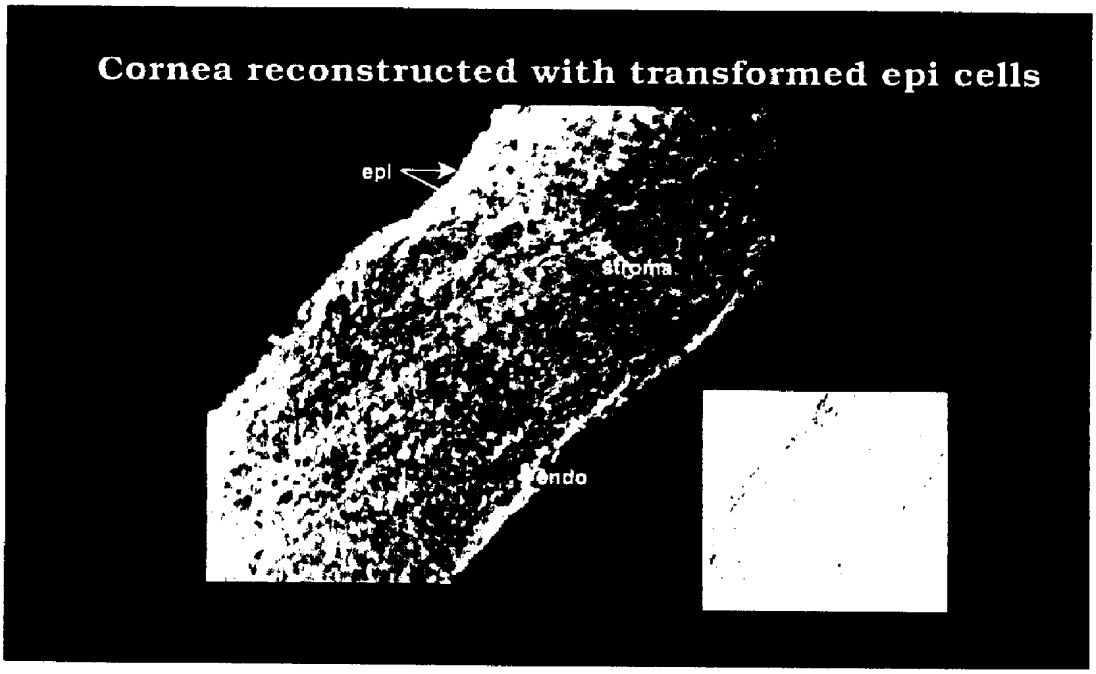

Fig. 6. A cornea that was reconstructed with cell lines that were transformed. The three main layers are not readily distinguishable and the transformed epithelial cells are seen invading the stromal compartment. This sample demonstrates the importance of obtaining immortalized cell lines that are physiologically similar to low passage primary cells from the human cornea. Inset: H&E section.

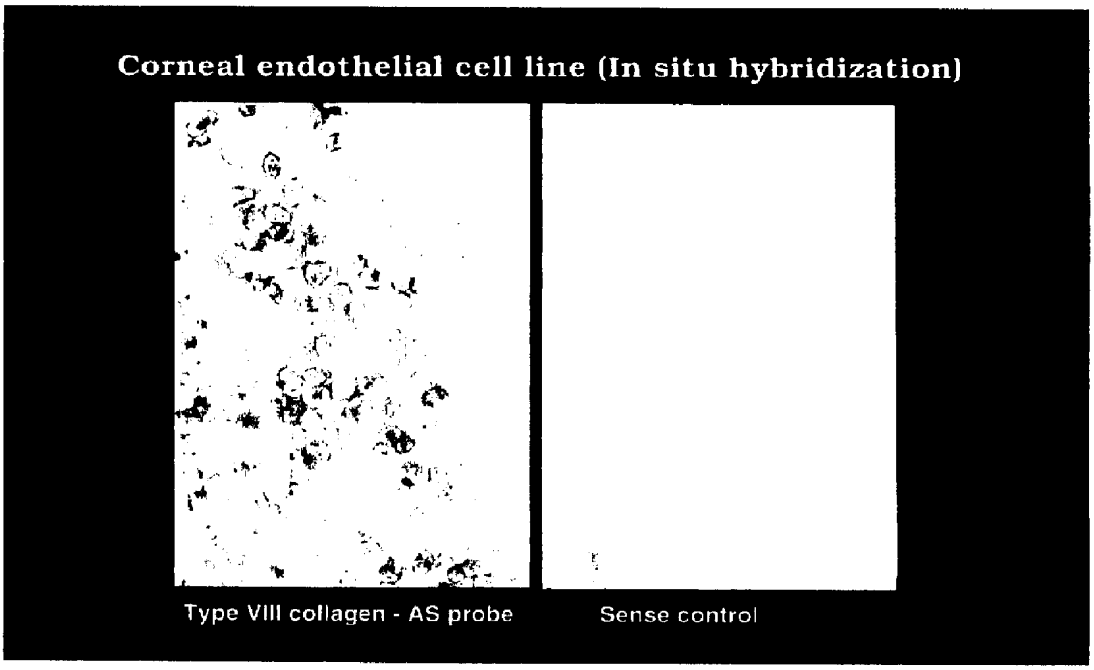

Fig. 7. In situ hybridization , using a probe for alpha2(VIII) collagen, which is found in Descemet's membrane in the cornea and is known to be produced by corneal endothelial cells. Alpha2(VIII) mRNA is produced by this endothelial cell line, as shown by visualization of cell-bound alkaline phosphatase conjugated DIG-antisense riboprobe. The sense control-probe cells remain unlabelled.

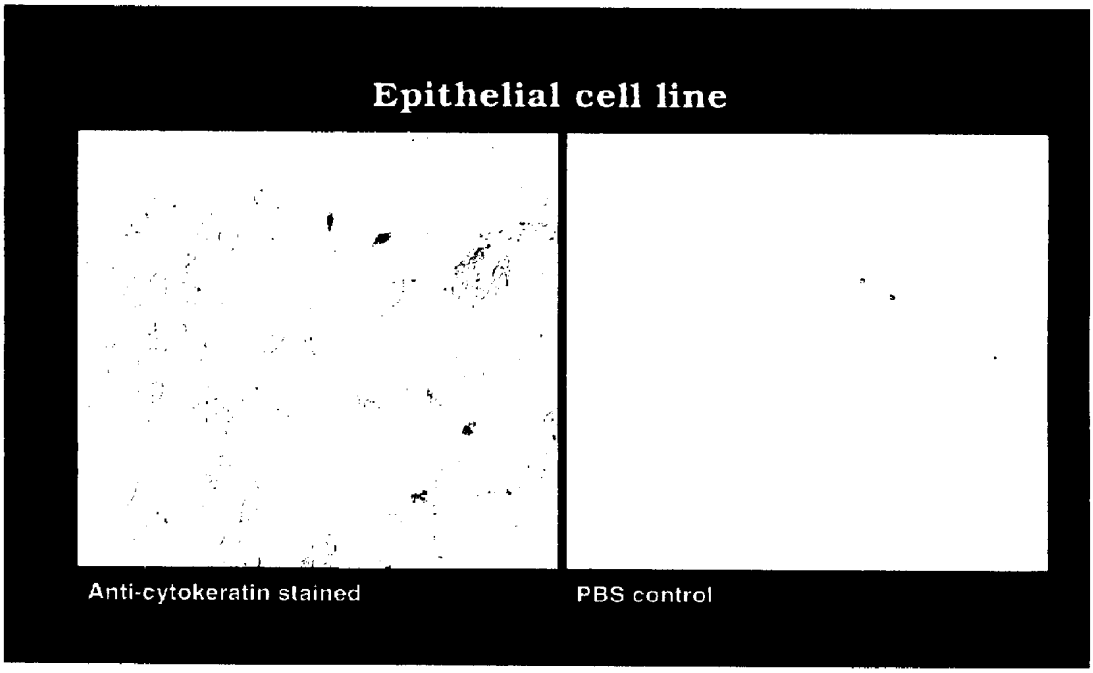

Fig. 8. Epithelial cell line showing expression of a standard marker, cytokeratin by immunohistochemistry.

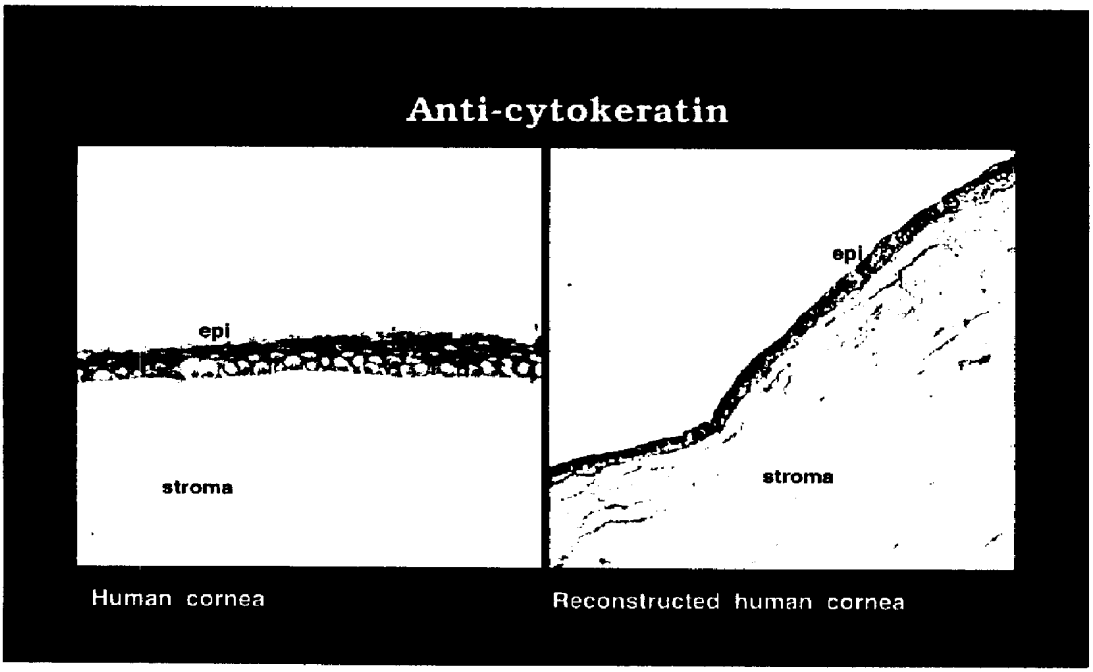

Fig. 9. Cytokeratin expression in the epithelium of a human cornea and in a reconstructed cornea.

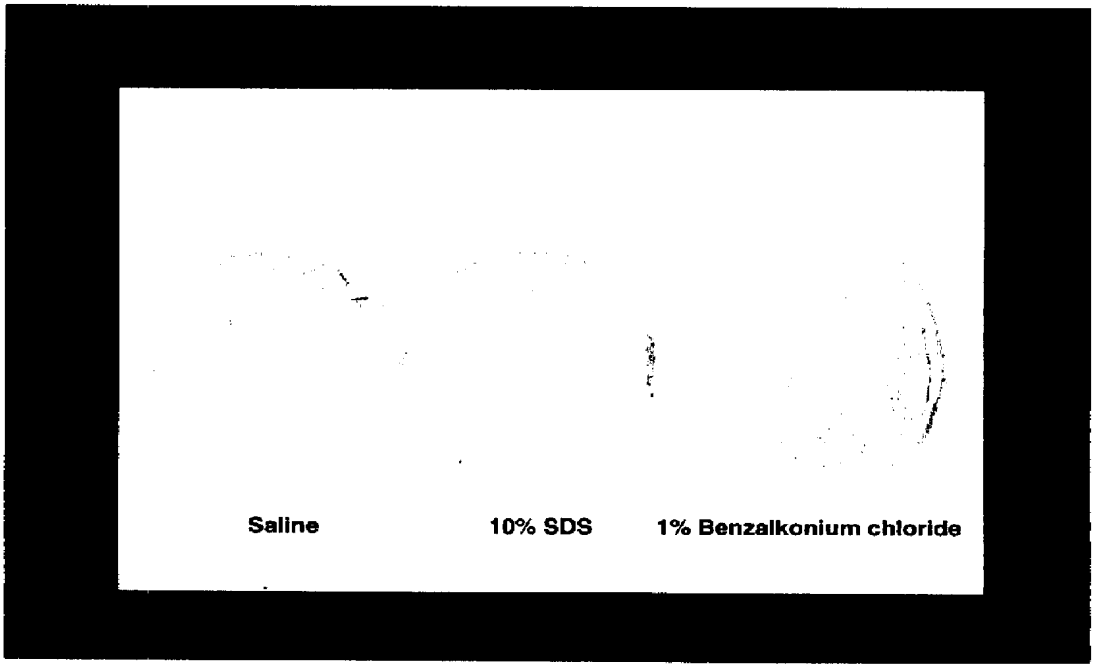

Fig. 10. Results from an initial functionality test of the reconstructed corneas. Each cornea was dosed with 100 µl of a test chemical delivered as an aqueous solution for 10 min., after which the chemical was washed off with saline and the corneas were re-cultured for a further three days. The saline-treated cornea served as the control. Treatment with SDS and benzalkonium chloride caused wounds to the cornea that are seen in this figure as changes in corneal transparency.

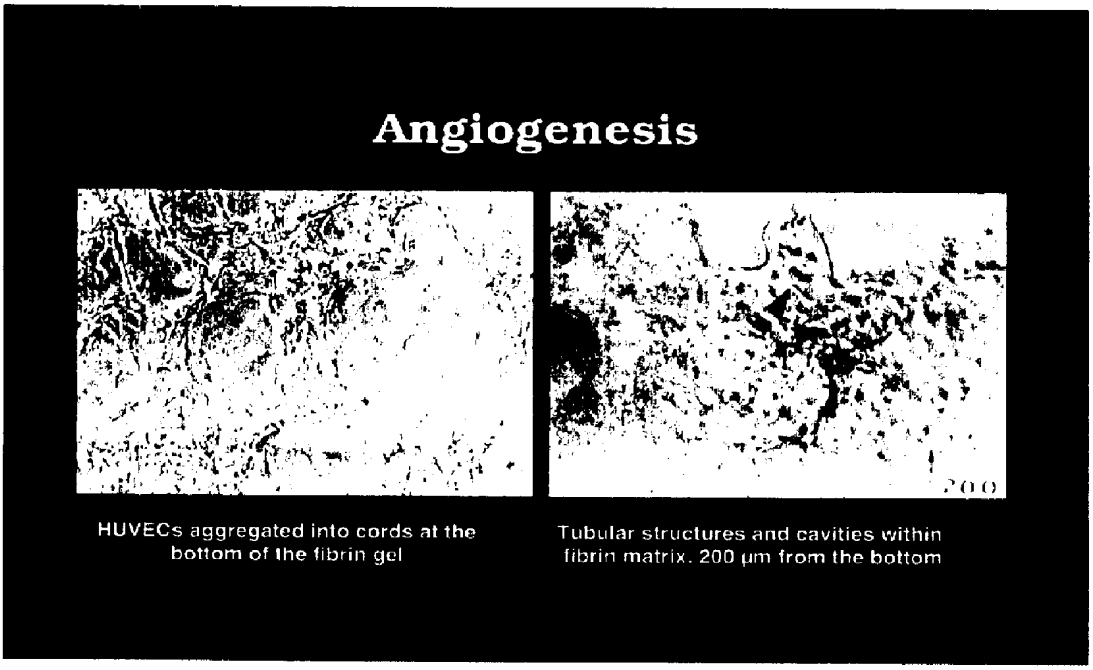

Fig. 11. Phase contrast micrographs showing developing capillary-like structures within the fibrin matrix of the "sclera".

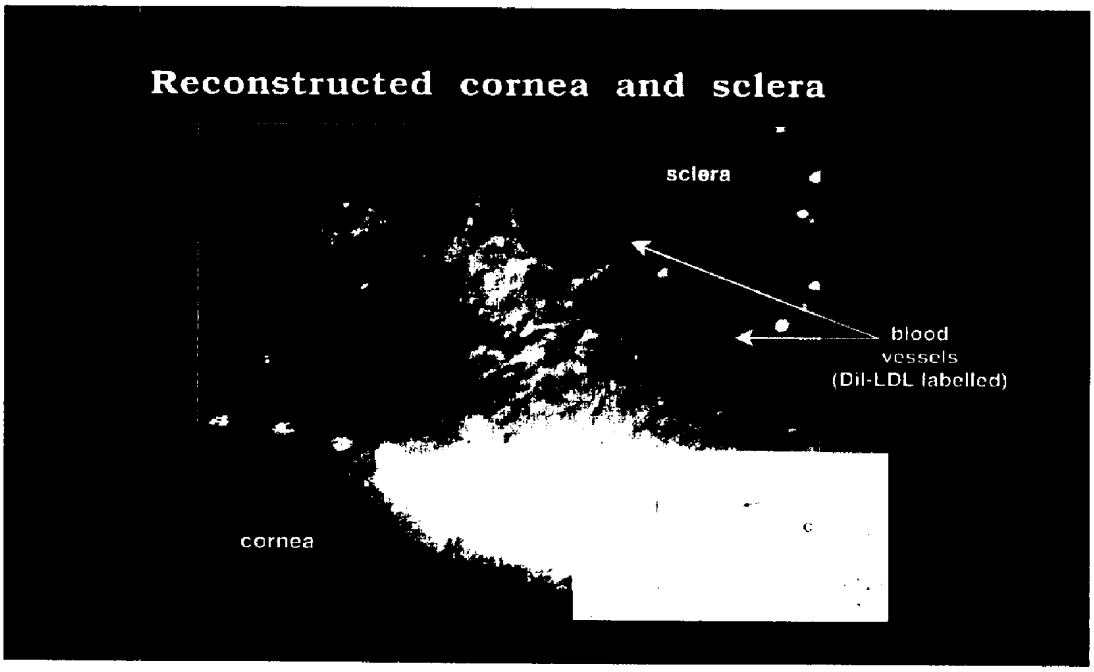

Fig. 12. Reconstructed human cornea and sclera. The angiogenic cells of the sclera have been labelled with a fluorescent marker, DiI-conjugated Low Density Lipoprotein (DiI-LDL). Inset: H&E stained cornea and sclera. f, fibrin matrix with blood vessels; c, cornea. The cornea-sclera junction is indicated by the arrow.

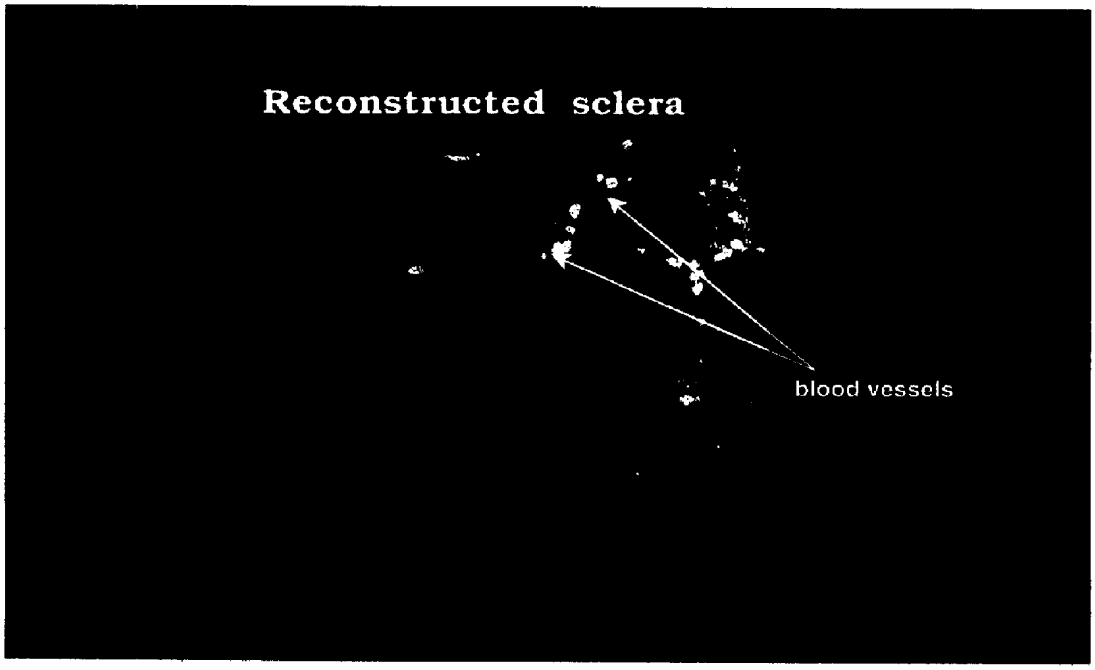

Fig. 13. Higher magnification of the sclera showing Di-I LDL labelled blood vessels.

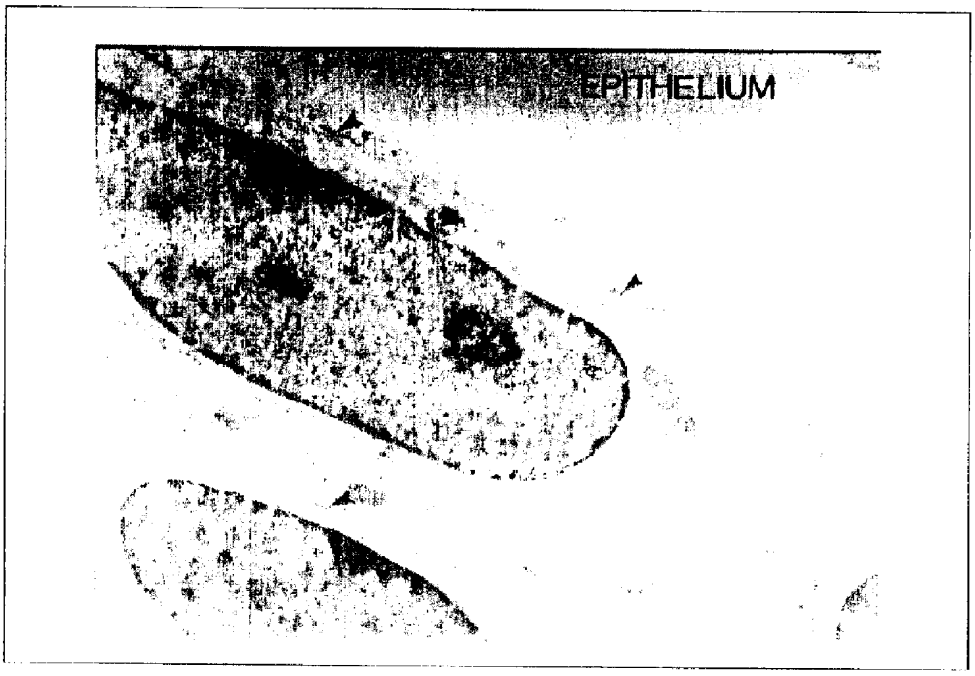

Fig. 14. Transmission electron micrograph of the epithelium of a reconstructed cornea after two weeks of culture at an air-liquid interphase. Microplicae, a characteristic of corneal epithelial cells are present on the external surface and junctional complexes have formed between cells (arrowheads).

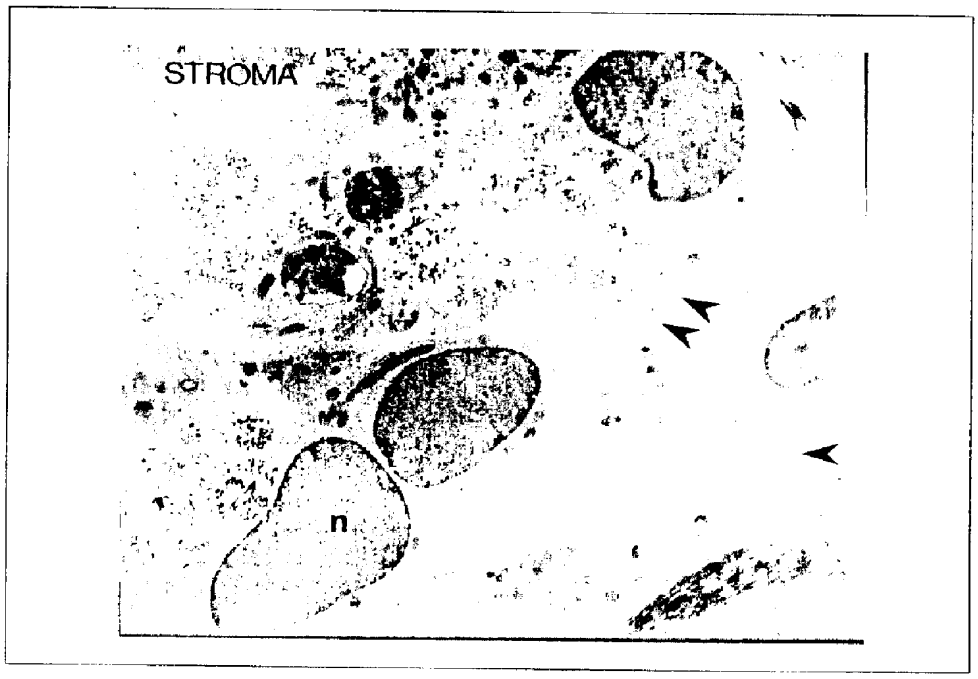

Fig. 15. Transmission electron micrograph of a reconstructed cornea after two weeks of culture at air-liquid interphase, showing stromal keratocytes within a largely collagenous matrix. The nuclei (n) are electron lucent, and there are mitochondria (arrowheads) in the cytoplasm, indicating that the cells are healthy.

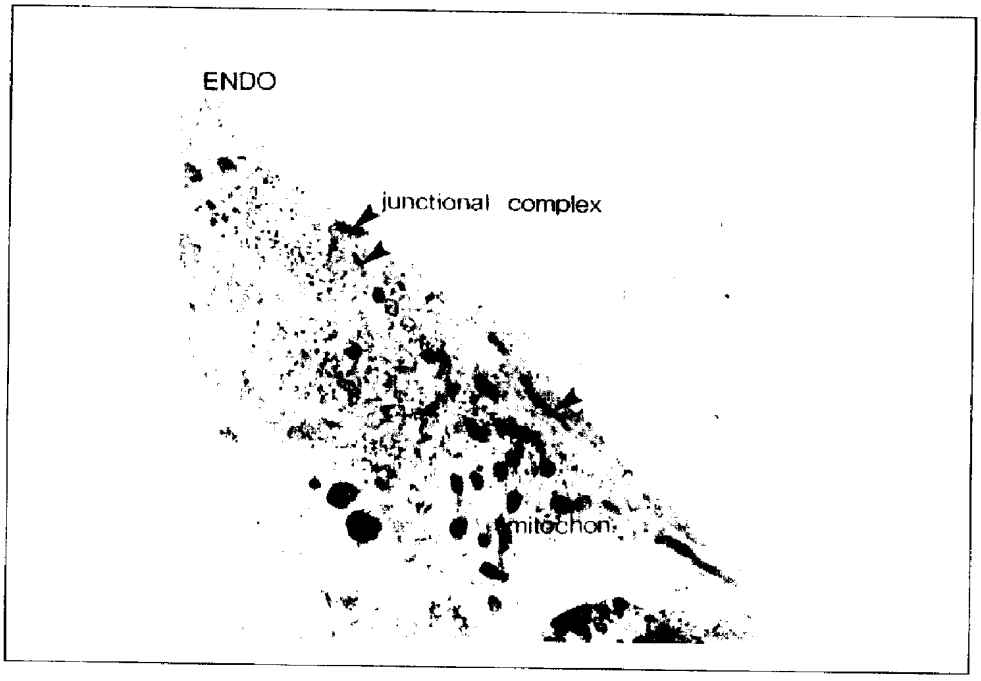

Fig. 16. Transmission electron micrograph of the endothelial layer of a reconstructed cornea after two weeks of culture at air-liquid interphase. Mitochondria are present in the cytoplasm as indicated, and junctional complexes (arrowheads) have formed between cells.